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THE SIGNIFICANCE OF METABOLIC CHANGES IN EASTERN WHITE PINE SEEDLINGS FOR EARLY DIAGNOSIS OF FLOURIDE INJURY

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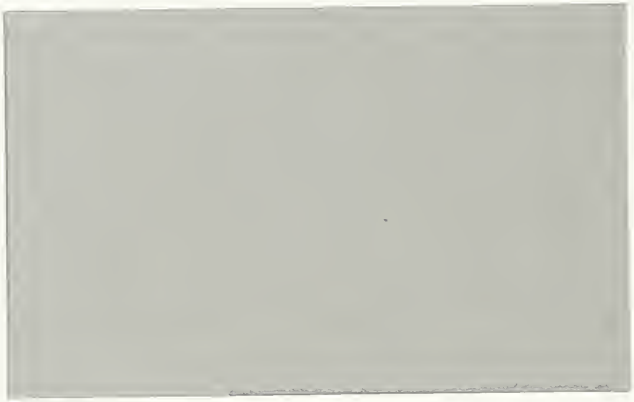

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THE SIGNIFICANCE OF METABOLIC CHANGES IN EASTERN WHITE PINE SEEDLINGS FOR EARLY DIAGNOSIS OF FLOURIDE INJURY

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Executive Summary

This study was funded by the Ontario Ministry of the Environment (MOE), Research and Technology Branch, project number 483G. The study investigated early metabolic changes in eastern white pine seedlings in response to relatively low concentrations of hydrogen fluoride (HF). The concentrations used in this study (0.5 ppb HF and 2 ppb HF) and durations of exposure (1 to 28 days) represent levels ranging from just below to just above the current Ontario fluoride (F) air quality criterion (0.5 ppb/30 days, and 1 ppb/24 hours). The report is formatted in journal style in two chapters, each dealing with separate analytical phases of the study. The authors intend to submit each chapter as separate papers for publication in a peer-reviewed scientific journal.

The results of the study indicated that the initial metabolic injury to eastern white pine seedlings occurred within 48 hours following the exposure to HF. Two days after the initiation of F treatments sterol:phospholipid levels dramatically increased in the plasma membranes of actively growing plants treated with 0.5 ppb and 2 ppb HF. Increased sterol:phospholipid ratios are known to reduce fluidity and increase leakiness in both plant and animal cells. This was corroborated by the leakiness of membranes that was observed in eastern white pine seedlings treated with HF fluoride for 2 days. Changes in membrane permeability may explain why a number of different nonspecific physiological effects of F on plants have been reported in the literature.

Light and electron microscopy do not offer adequate sensitivity to discern changes in cells of seedlings before visible damage occurs. In these experiments, the first signs of visible F injury (needle tip necrosis) were observed after 20 days in 0.5 ppb HF treatment and 5 days in 2 ppb treatment.

Visible foliar injury by HF to eastern white pine seedlings can occur at ambient concentrations in the range of the MOE current F air quality criterion. Metabolic injury occurred even earlier than the visible injury. Fumigation with HF at concentrations as low as 0.5 ppb for as short as 2 days resulted in an increase in the sterol:phospholipid ratio of the plasma membrane. This resulted in increased membrane permeability and a subsequent increase in electrolyte leakage. This was concurrent with increased ATPase activity, presumably to fuel metabolic repairs. These metabolic effects were concurrent with decreased foliar transpiration resulting in increased foliar water potential. Therefore, short-term exposure to low levels of HF may actually improve the plant's drought resistance. However, chronic exposure to low concentrations of HF may result in the plant expending more energy but fixing less carbon (a reduction in net photosynthesis), because of reduced stomatal function inhibiting CO_2 intake and enhanced ATPase activity associated with membrane damage and repairs. It was beyond the scope of this study to establish the impact of chronic HF exposure on seedling performance and to determine whether these early metabolic changes are reversible.

Actively growing eastern white pine seedlings appear to be very sensitive to F pollution and the results of the present study support their use for early detection of F damage. Plasma membranes appear to be among the initial sites of F injury and can be used to detect early F effects. Plasma membranes of white pine seedlings are adversely affected by ambient HF concentrations in the range of the current Ontario air quality criterion (0.5 ppb/30 days).

CHAPTER I.

EARLY EFFECTS OF HYDROGEN FLUORIDE ON WATER RELATIONS, PHOTOSYNTHESIS AND MEMBRANE INTEGRITY IN EASTERN WHITE PINE (PINUS STROBUS) SEEDLINGS.

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ABSTRACT

Eastern white pine (Pinus strobus L.) seedlings were treated with 0.5 and 2 ppb hydrogen fluoride (HF) for 1 to 28 days and water relations, photosynthesis and membrane integrity were studied to determine the mode of fluoride (F) action on plants. The first symptoms of F injury appeared on young needles as tip necrosis after 20 days of 0.5 ppb HF treatment and 5 days of 2 ppb HF treatment. Both HF concentrations reduced transpiration rates in seedlings treated for 1, 7 and 27 days, but had little effect on net photosynthesis. The initial decline in transpiration rates was not accompanied by an increase in tissue F levels. Reduced transpiration was a possible cause of higher water potentials and water contents in F-treated seedlings compared with control plants.

Changes in membrane permeability were among the earliest noticeable effects of HF and greater electrolyte leakage was observed from seedlings treated for 2 and 8 days with HF compared with controls. The results of this study suggest that cell membranes are among the initial sites of F action on plants.

INTRODUCTION

Fluoride (F) is a common industrial air pollutant released during various industrial operations such as those producing steel, aluminum, ceramics and phosphate fertilizers. Average atmospheric F concentrations vary from less than 0.1 ppb in non-urban areas to over 2.3 ppb in urban and industrial areas (1). Fluoride is considered to be the most phytotoxic of all common air pollutants (12). Fluoride accumulates in plants and it has been believed that plant injury occurs when a certain threshold tissue concentration is reached (2). However, the exact mechanism of F action on plants remains unclear. Fluoride is highly reactive and in many in vitro systems it acts as a potent enzyme inhibitor. It is possible that F alters metabolic processes directly by influencing enzyme activity, however, very few in vivo studies demonstrated a significant effect of F on enzymes. Fluoride is known to alter various processes in plants including photosynthesis, respiration, as well as carbohydrate, lipid and nucleic acid metabolism (11). This suggests the involvement of secondary F effects triggered by the initial plant response.

Some of the observed changes in F-treated plants could be attributed to water stress which was found to be induced in jack pine seedlings by soil F (17, 18). On the other hand, high concentrations of air F were shown to increase the water potentials in soybean (8). Zwiazek and Shay (17, 18) observed numerous membrane changes in soil F-treated plants. However, these changes could not be separated from those in water-stressed plants and it is not clear whether they represent a direct response to F.

The principal objective of the present study was to examine early changes in eastern white pine seedling in response to low air F concentrations. In the present paper we report the effects of low concentrations of HF (hydrogen fluoride) water relations, gas exchange and membrane permeability in eastern white pine seedlings. Eastern white pine was selected for this study because of its high sensitivity to F and it's presence in many sites close to industrial F sources.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Eastern white pine (Pinus strobus L.) seeds from a seed source representative of central Ontario were obtained from the Provincial Seed Tree Plant, Angus, Ontario, Canada. The seeds were germinated and seedlings grown in plastic containers (20 cm x 3.8 cm, length x diameter) containing a mixture of peat-vermiculite (2:1). The containers were placed in a greenhouse with daylight supplemented to 18 hours by 400-W high pressure sodium lamps. The seedlings were fertilized weekly with 229-29-154 commercial fertilizer supplemented with iron.

Nine-week-old seedlings were treated with HF in 39 x 75 x 47-cm plexiglass fumigation chambers that were placed in controlled environment growth cabinets. The following conditions were maintained inside the fumigation chambers during the experiments: day/night temperature, 22/15°C; relative humidity, 85+5%; 18-h photoperiod; 200+10 uM m^{-2} s^{-1} photon flux density. Seedlings were watered twice a week to runoff. Treatments included 0 (control), 0.5 and 2 ppb HF for 1 to 28 days. Hydrogen fluoride was introduced into the chambers by passing compressed air through 1-l polyethylene bottles each containing 500 ml of deionized water (control), 0.005% hydrofluoric acid (0.5 ppb treatment) or 0.02% hydrofluoric acid (2 ppb treatment) (13). The total rate of air flow through the chamber was 2.5 l min^{-1} (0.5 l min^{-1} passing through the fumigation bottle and 2 l min^{-1} ventilation flow). Air flowing out of the chamber was passed through a 0.5-l polyethylene bottle containing 250 ml of 1.0 N NaOH. Fluoride trapped in this solution was measured as described by McQuaker and Gurney (7) to monitor HF concentration inside the chambers. For all measurements plants without apparent F injury (needle tip necrosis) were selected and the entire experiment was repeated.

Measurements of Photosynthesis, Water Relations and Fluoride Content

Net photosynthesis in F-treated and control seedlings was measured inside the fumigation chambers using a portable infra-red gas analyzer LCA-3 (Analytical Development Corporation, Hoddesdon, U.K.) and transpiration rates were measured using a LI-1600 (Li-Cor, Lincoln, Nebrasca, U.S.A.) steady state porometer. Needle areas for photosynthesis and

transpiration were determined using a Li-Cor 3100 area meter.

Osmotic potentials were measured in needles in a dew point mode using an HR-33T dew point microvoltmeter (Wescor, Logan, Utah, U.S.A.) equipped with triple psychrometer chambers (Plant Water Stress Instruments, Guelph, Ontario, Canada). Needles were placed in aluminium foil envelopes containing small circles of Whatman 1 filter paper. The envelopes were immersed in liquid nitrogen and placed at -17°C for 24 h. After thawing, the needles were crushed in a vise and the osmotic potentials of sap-saturated circles were measured (14).

Water potentials were measured in shoots using a pressure chamber technique (9) as previously described (15) and the water content was estimated in oven-dried needles at 85°C for 24 hours.

Fluoride content of needles was determined using an alkali fusion-selective ion electrode technique of McQuaker and Gurney (7). Each sample consisted of needles combined from two seedlings. The needles were dried at 105°C for 24 hours and pulverized. The samples containing about 0.25 g of pulverized needles were transferred to 50-ml nickel crucibles. To each sample 3 ml of 67% NaOH was added and the crucible was placed in an oven at 150°C for 1 h and cooled. After NaOH had solidified, the crucible was placed in a muffle furnace set to 300°C. The temperature was then raised to 600°C and the sample was fused at this temperature for 30 min. After the crucible had been removed from the muffle furnace and allowed to cool, 5 ml of distilled water was added to the sample followed by 4 ml of concentrated HCl to adjust the pH to 8-9. Once the acidified sample had cooled, it was transferred to a 50-ml volumetric flask, diluted to volume, and then filtered through dry Whatman No. 40 filter paper. Fluoride concentration was determined potentiometrically using a F-selective electrode (Orion Research Inc., Boston, MA, U.S.A.) after adjusting pH of the solutions to 5.2.

Electrolyte Leakage

Electrolyte leakage from needles was determined by measuring electrical conductance of cell diffusates as described previously (15, 16). Needles (7 per sample) were briefly washed, cut into 1-cm segments, placed in test tubes containing deionized water, and incubated at 21°C.

After 1 hour the solutions were replaced with 5 ml of fresh deionized water and the incubation continued for 5 more hours. Electrical conductance of diffusates was measured with a Mdl C33 conductivity meter (Fisher Scientific Co., Ottawa, Ontario, Canada). After measurements, the needles were frozen and stored overnight at -17°C. Thawed needles were again incubated for 5 hours and the electrical conductance of cell diffusates was measured to determine the total electrolyte content of needles.

RESULTS

Seedling Fluoride Content and Visible Injury

The increase in needle Fluoride content was observed after 7 days in 2 ppb and after 23 days in 0.5 and 2 ppb HF-treated plants. Shorter treatment duration did not affect tissue F levels (Fig. 1).

The initial signs of F injury appeared on young needles after 20 days in 0.5 ppb and 5 days in 2 ppb of HF treatments. In both treatments some seedlings developed tip necrosis that progressed basipetaly. In many needles reddish-brown succession bands across the brown necrosis was observed.

Water Relations and Photosynthesis

Transpiration rates drastically declined in seedlings treated with 0.5 ppb and 2 ppb HF for 1 day. In plants treated for 7 days with 0.5 ppb HF transpiration rates remained lower than those in controls, but the transpiration of 2 ppb treated seedlings and control plants did not differ significantly. Both F concentrations decreased transpiration rates in seedlings treated for 27 days (Fig. 2a).

Hydrogen fluoride treatments had little effect on net photosynthesis in seedlings. A slight decrease in photosynthetic rates was observed in seedlings treated with 0.5 and 2 ppb HF for 1 and 7 days followed by an insignificant increase in seedlings treated for 27 days (Fig. 2b).

Hydrogen fluoride treatment resulted in an increase in water potentials after 7 and 27 days in 0.5 ppb treatment and after 7 days in 2 ppb treatment. However, plants treated with both F concentrations for 7 and 27 days had similar water potentials to those in control seedlings (Fig. 2c).

Osmotic potentials were higher in plants treated with 0.5 ppb HF for 7 and 27 days compared with controls and a slight decrease was observed in 2 ppb treated seedlings for 1 and 7 days (Fig. 2d).

Water contents increased in seedlings subjected to the 0.5 ppb treatment for 1, 7 and 27 days compared with untreated plants. However, little effect of HF on water content was seen in the 2 ppb treatment (Fig. 2e).

Electrolyte Leakage

Hydrogen fluoride treatments resulted in an increased leakiness of electrolytes (Fig. 3). Plants treated with 2 ppb HF for 2 days leaked about 50% more electrolytes compared with control seedlings. Electrolyte leakage was also higher in plants treated with 0.5 and 2 ppb HF for 8 days, but there was little difference in the extent of electrolyte leakage between control seedlings and plants treated with HF for 28 days (Fig. 3).

DISCUSSION

Decreased transpiration rates, elevated water potentials and water content and increased electrolyte leakage were among the earliest changes observed in HF treated eastern white pine seedlings. It is possible that the observed increase in water potentials and water content resulted from F-induced stomatal closure. Poovaiach and Wiebe (8) showed that guard cells in soybean are the first to be affected by gaseous F resulting in improved water relations. On the other hand, soil F was shown to induce water stress in jack pine seedlings, probably by suppressing stomatal movements (17, 18). Zwiazek and Shay (18) discussed the possibility that water stress induced in plants by F may be responsible for many of the observed physiological alternations. However, the results of the present study indicated that low concentrations of

gaseous HF did not produce water stress in eastern white pine seedlings. It appears that stomatal movements may be differently affected by different forms or concentrations of F with low concentrations of gaseous F promoting stomatal closure and high soil F concentrations promoting stomatal opening. It is interesting that the increased electrolyte leakage from seedlings was noticeable at the same time when reduced transpiration rates were observed. This could indicate changes in selective permeability of cell membranes including those in the guard cells. Electrolyte leakage was also shown to be among the earliest detectable effects in jack pine seedlings suffering from soil F-induced water stress (18).

In the present study we did not observe a significant effect of gaseous HF on photosynthesis. A slight decrease in net photosynthesis of seedlings could be largely explained by reduced stomatal conductance. Our findings support those of others (3, 5) suggesting that despite apparent sensitivity of thylakoid structures to F (4, 17), photosynthesis is not drastically affected by low doses of F.

It has been long known that F can affect plants at the metabolic level in the absence of visible injury (6, 10). It has been believed that plant injury occurs when a certain threshold tissue level is reached (2). Our results indicate that low concentrations of HF can alter physiological and biochemical processes long before detectable F accumulation in needles. In the present study we also noted some F accumulation in control seedlings. This was likely due to trace quantities of F present in unfiltered air passed through the fumigation chambers.

The mechanism of F action on plants is a complex one and involves numerous secondary changes that are difficult to separate from direct initial effects. We suggest that F initially affects plants by interfering with the selective permeability of cell membranes. As a result of cell membrane changes other processes in plants are affected leading to metabolic and visible injury. A recent study completed in our laboratory will provide evidence that the composition of plasma membranes and plasma membrane bound ATPase activity in eastern white seedlings are severely altered by a short exposure to HF.

ACKNOWLEDGMENT AND DISCLAIMER

Funding for this study was provided by the Ontario Ministry of the Environment in the form of a research grant to J.J. Zwiazek. The views and ideas expressed in this report are those of the authors and do not necessarily reflect the views and policies of the Ministry of the Environment, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Figure 1. Fluoride content in needles of eastern white pine seedlings subjected to HF treatments. Bars indicate means of four samples + SE.

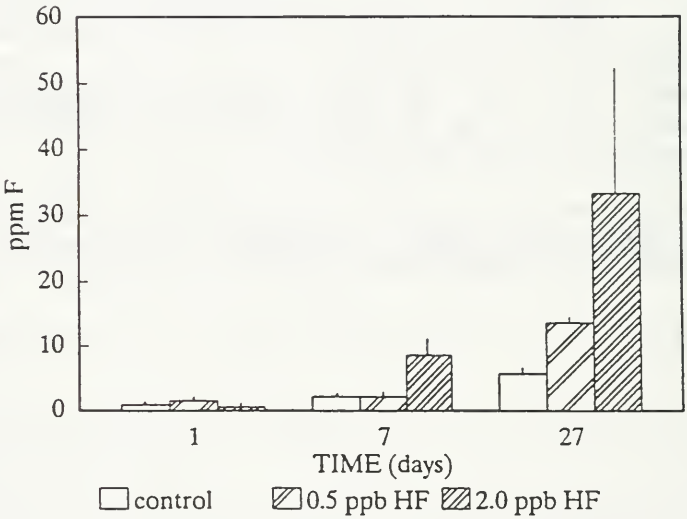


Figure 2. Effect of HF on (a) transpiration rates, (b) photosynthesis, (c) water potentials, (d) osmotic potentials and (e) water content in eastern white pine seedlings. Data are means of eight seedlings + SE.

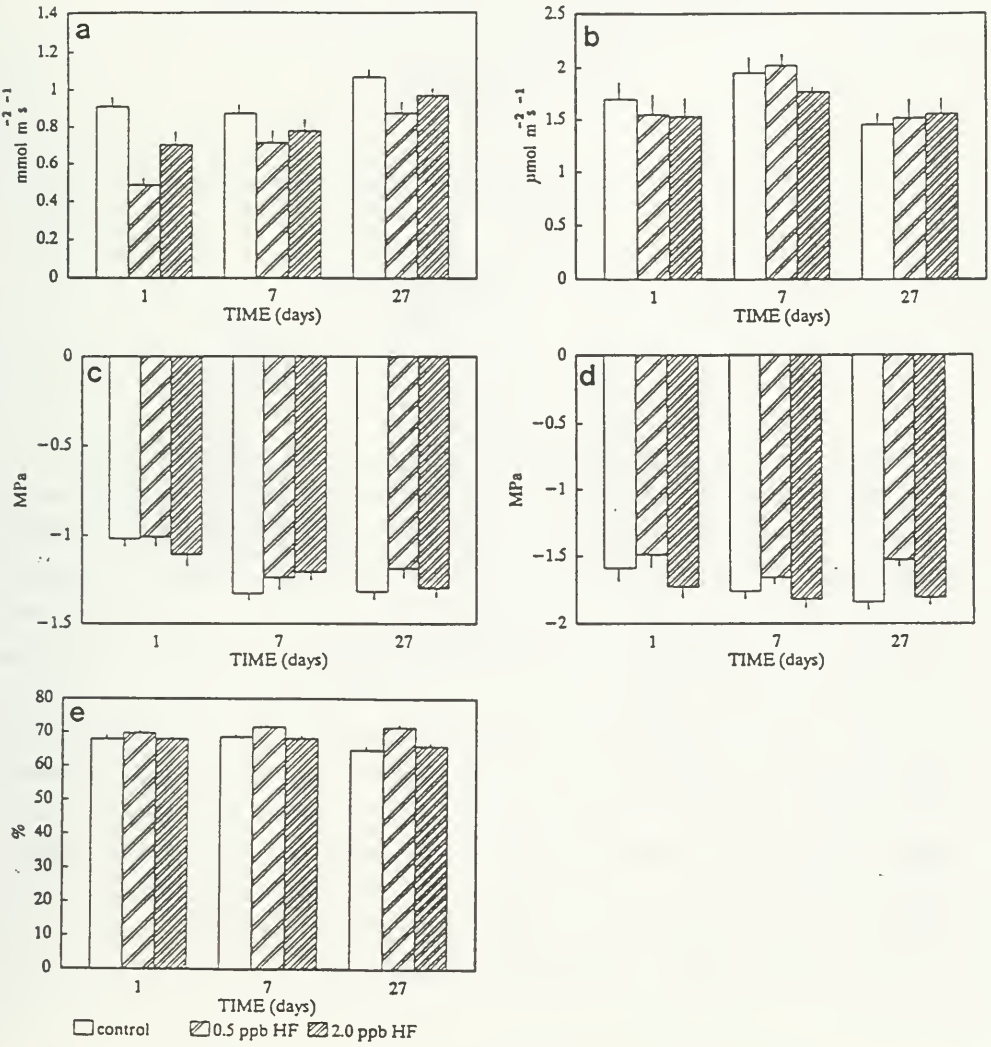
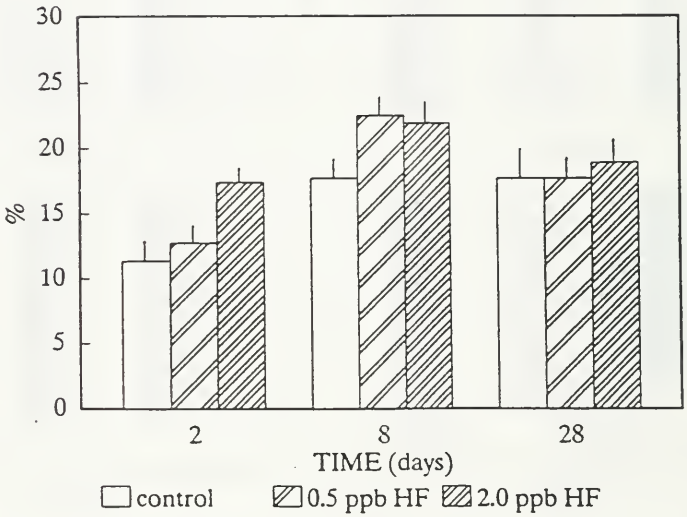


Figure 3. Effect of HF on electrolyte leakage in eastern white pine seedlings. Bars indicate means (n=5) + SE.



CHAPTER II.

HYDROGEN FLUORIDE EFFECTS ON PLASMA MEMBRANE LIPID COMPOSITION, ATPASE ACTIVITY AND CELL STRUCTURE IN NEEDLES OF GROWING AND DORMANT EASTERN WHITE PINE (PINUS STROBUS) SEEDLINGS.

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ABSTRACT

Eastern white pine (Pinus strobus L.) seedlings grown under long day conditions (actively growing) and short day conditions (dormant) were fumigated with 0.5 and 2 ppb hydrogen fluoride (HF) for 2-28 days. Plasma membranes were isolated from needles of treated and untreated seedlings and their lipid composition and ATPase activity examined to determine early effects of fluoride (F) action. In growing seedlings plasma membrane-free sterol levels (campesterol, stigmasterol and sitosterol) increased after 2 days in both F treatments. In dormant plants an increase in oleic and linoleic acids was observed in phospholipids after 2 days of 0.5 ppb HF treatment. Plasma membrane ATPase activity in growing seedlings increased after 8 days in both F treatments. In dormant plants an increase of ATPase activity was observed after 28 days in 2 ppb HF treatment. No differences in mesophyll cells structure were noticeable in F-treated plants before the appearance of visible injuries. The results of this study indicate that changes in the plasma membrane lipid composition and ATPase activity of growing seedlings occur during early stages of F injury. Longer durations of F treatment are required to induce similar changes in dormant plants.

INTRODUCTION

Fluoride is considered to be the most phytotoxic of all common air pollutants (Weinstein 1977). Our earlier study (Rakowski and Zwiazek 1992) indicated that hydrogen fluoride (HF) can affect membrane integrity, water relations and transpiration rates several days before the appearance of visible needle injury and before an increase in F tissue content can be detected. The results of this study suggested an early response of cell membranes to fluoride pollution.

Fluoride is a potent in vitro inhibitor of ATPase activity, including plasma membrane ATPase (Giannini et al. 1987). However, plasma membranes and plasma membrane ATPase activity has not been previously analyzed in F-treated plants.

Rapidly growing and young saplings are frequently more sensitive to pollution stress than dormant and older trees (MacLean et al. 1986). However the exact cause of this different response is not clear.

In the present study we examined the effects of low concentrations of HF on plasma membrane lipid composition and ATPase activity in needles from growing and dormant eastern white pine (Pinus strobus L.) seedlings. The objective of this study was to determine differences in the response of plasma membranes to F in actively growing and non-growing seedlings and evaluate the possible use of the plasma membrane analysis for early detection of F injury.

MATERIALS AND METHODS

Plant Material and Experimental Conditions.

Eastern white pine (Pinus strobus L.) seeds were germinated and seedlings grown in the greenhouse as previously described (Rakowski and Zwiazek 1992).

One group of seedlings was grown for 20 weeks under long day conditions (18-h photoperiod) to induce high growth and photosynthetic rates. Seedlings of the second group

were grown for 12 weeks under long day conditions and then dormancy was induced by placing them for 4 weeks under short day (12-h photoperiod) conditions. This treatment resulted in an inhibition of photosynthesis and growth.

Twenty-week-old seedlings were treated with 0.5 ppb and 2 ppb HF for 2 to 28 days in plexiglass fumigation chambers as described earlier (Rakowski and Zwiazek 1992). For all analyses, only healthy-looking seedlings without needle necrosis were taken.

Seedlings used for electron microscopy were grown for nine weeks under long day conditions and then treated with 0.5 and 2 ppb HF for 1 to 23 days.

Plasma Membrane Isolation

Fresh needles (20 g) were frozen in liquid nitrogen, crushed and homogenized with 100 ml of the extraction medium containing 1% PVPP and 0.16 ml mercaptoethanol in 0.75 M Tris-acetate buffer at pH 7.0. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 600 g for 15 min. The pellet was discarded and the supernatant was centrifuged at 70 000 g for 1 h. The pellet obtained as a result of this centrifugation was resuspended in 5 ml of a medium containing 0.5 M sucrose and 10 mM sodium phosphate (pH 7.8) and used for phase partitioning against 6.3% 3350 polyethylene glycol and 6.3% dextran 500 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Phase partitioning was carried out according to Hellergren et al. (1983).

Plasma Membrane Lipid Analysis

Lipids from the plasma membranes were extracted with acetone:ethanol (1:1, v:v) followed by chloroform:methanol (2:1, v:v) and chloroform. All solvents were evaporated and lipids resuspended in a small volume of chloroform to which internal standards of n-cholestane and posphatidylcholine diheptadecanoyl were added and applied to a silicic acid column (0.5 x 5 cm, 100-200 mesh). The lipids were separated into different polarity classes by washing the column with 10 column volumes of chloroform, followed by 40 volumes of acetone, 10 volumes of chloroform:methanol (1:1, v:v) and 10 volumes of methanol. The chloroform fraction contained sterols, the acetone fraction contained mostly galactolipids, and the chloroform:methanol and methanol fractions contained phospholipids (Zwiazek and Shay

1988).

The fraction containing sterols was evaporated under nitrogen, dissolved in 0.5 ml of pyridine and trimethylsilyl derivatives of sterols were made by adding 0.1 ml trimethylchlorosilane followed by 0.2 ml hexamethyldisilazane (Zwiazek and Blake 1990). Sterols were analyzed using a Hewlett Packard 5890 gas chromatograph equipped with a 30-m-long, 0.25-mm internal diameter DB-5 column (J&W Scientific, Folsom, CA, U.S.A.). Conditions of the analyses were: carrier gas (helium) linear flow, 33 cm s^{-1}; oven temperature, 285°C; detector and injector temperatures 300°NEC Silentwriter LC-890NESILC89.PRSroscopy (GC-MS) analysis. GC-MS analysis was performed using a VG 7070E mass spectrometer, EI+ mode, 70 eV.

The fractions containing phospholipids were combined, dried under nitrogen and phospholipids were suspended in 0.5 ml of chloroform. Phospholipids were transestrified using 0.5 M sodium methoxide and fatty acid methyl esters were analyzed using a Hewlett Packard 5890 gas chromatograph equipped with a 30-m long, 0.25-mm internal diameter DB-225 column. Conditions of the analyses were: carrier gas (helium) linear flow, 15 ml min^{-1}; oven temperature, 200°C; detector and injector temperatures, 250oC. The identification of phospholipids was confirmed by cochromatography with authentic standards.

<u>Measurements of Plasma Membrane ATPase Activity</u>

Plasma membrane ATPase activity was determined in a reaction medium containing of 10-20 ug membrane protein, 3 mM ATP, 4 mM $MgSO_4$, 50 mM KNO_3, 1 mM sodium azide, 0.1 mM sodium molybdate, 50 mM MES-Tris (pH 6.5), 0.33 M sucrose, 0.1 mM EDTA, 1 mM DTT, 0.05% Triton X-100 and + 0.1 mM orthovanadate. Orthovanadate was stored as 5 mM vanadate solution and prepared by dissolving V_2O_5 in 20 uM NaOH at pH 11.8 (Gallagher and Leonard 1982). The reaction was carried out at 38°C and stopped after 30 min by adding 1 ml of ascorbate/molybdate solution containing 3% (w/v) ascorbic acid, 1% (w/v) sodium dodecyl sulfate (SDS), 0.5 M HCl and mixed 15 min before use with freshly made 8% (w/v) ammonium heptamolybdate in a ratio of 10:1. Released inorganic phosphorus (Pi) was estimated using the method described by Widell and Larsson (1990). The protein content of the plasma membranes was estimated by a modified micro-Lowry method (Peterson 1977)

using bovine serum albumin as a standard.

Microscopy

For electron microscopy and light microscopy, approximately 1-mm-long segments were excised from the centre of juvenile needles. The segments were fixed at room temperature overnight in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.9) and postfixed in 1% osmium tetroxide in phosphate buffer for 8 h at 4°C. After fixation, the blocks were dehydrated in an ethanol series and transferred to propylene oxide and then to mixtures of propylene oxide and Spurr's resin (1:2, 1:1, 2:1). Propylene oxide was evaporated under a fume hood and the tissue segments were infiltrated for 7 days in Spurr's resin. The resin was polymerized overnight at 70° C and sections cut for light and electron microscopy. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and for light microscopy with crystal violet (Zwiazek and Shay 1987).

RESULTS

Lipid Composition

A large increase in the ratios of sterols:phospholipids was observed in the plasma membranes of growing seedlings treated with 0.5 ppb and 2 ppb HF for 2 days (Fig. 1a). A smaller increase was also noted in 2-ppb-treated dormant plants after 2 days of HF treatment (Fig.1b). After 8 days and 28 days of HF treatments there were no differences in sterol:phospholipid ratios between F-treated and control actively growing plants (Fig.1a). However, the plasma membranes of HF treated dormant seedlings contained higher sterol:phospholipid ratios after 8 days (0.5 ppb and 2 ppb treatments) and 28 days (0.5 ppb treatment) (Fig.1b).

The principal sterols in the plasma membranes of eastern white pine seedlings were identified by GC-MS as B-sitosterol, campesterol and stigmasterol. The content of these sterols increased dramatically in growing seedlings treated for 2 days with both F concentrations (Fig.2a). A small increase in sitosterol and campesterol were also observed in growing seedlings after 8 days of F fumigation, but similar sterol levels in treated plants and controls were found after 28 days of HF treatment (Fig.2b, 2c).

Hydrogen fluoride had little effect on sterol content and composition in dormant plants treated for 2 and 8 days (Fig 3a, 3b). However, Seedlings subjected to the 0.5 ppb treatment for 28 days showed a several-fold increase in plasma membrane sterols compared with controls (Fig. 3c).

ATPase Activity

In growing seedlings plasma membrane ATPase activity slightly decreased after 2 days of 0.5 ppb HF treatment (Fig. 4a). After 8 days an increase in ATPase activity was observed in both HF treatments (Fig. 4a) followed by a drastic inhibition of the activity in 2 ppb treatment after 28 days (Fig. 4a).

Plasma membrane ATPase activity in dormant plants was not changed by F treatment for 2 days (Fig. 4b). A slight decline in ATPase activity was observed in seedlings treated for 8 days and about two-fold increase in plants subjected to 2 ppb HF for 28 days (Fig. 4b).

Cell Structure

Light microscopy failed to show significant changes in cell structure related to HF treatments. Fig. 5 shows needle structure of control (Fig. 5a), 0.5 ppb treatment for 23 days (Fig. 5b) and 2 ppb treatment for 7 days (Fig. 5c) viewed with a light microscope. The structure of needle mesophyll cells in control and F-treated seedlings did not appear to differ significantly when studied with an electron microscope. Fig. 6a shows the structure of control cells containing a large central vacuole and numerous chloroplasts with starch grains. Membrane morphology did not appear to be affected by 0.5 ppb treatment for 23 days (Fig. 6b) and 2 ppb treatment for 7 days (Fig. 6c). In both treatments starch grains were abundant in the chloroplasts and lipid bodies were occasionally seen in both F treatments and control cells.

DISCUSSION

Numerous studies have reported effects of F on physiological processes in plants (Miller and Miller 1974, Ballantyne 1972, Psenak et al. 1977, Lorenc-Plucinska and Oleksyn 1982, Weinstein and Alscher-Herman 1982 and others). However, the mechanisms of F action on plants remain unclear. Recent studies pointed to cell membranes as a possible initial site of F injury (Zwiazek and Shay 1987, 1988, Rakowski and Zwiazek 1992).

The plasma membranes are important structures in cells regulating transport processes. The plasma membranes of higher plants contain a H^+-ATPase as their major ion pump (Palmgren 1991). This enzyme plays a central role in plant nutrition and growth (Palmgren 1991). H^+-ATPase from sugar beet has been shown to be inhibited in vitro by F (Giannini et al. 1987). Plasma membrane lipids including sterols can regulate the activity of the H^+-ATPase (Palmgren et al. 1990, Grandmougin et al. 1989a,b, Sandstrom and Cleland 1989, Memon et al. 1989). Thus, the in vitro effect of F on enzyme activity could be modified by the enzyme lipid environment.

In the present study, changes in sterol composition and sterol:phospholipid ratios were dramatically changed in the plasma membranes of growing seedlings treated for 2 days and in dormant seedlings treated for 28 days. ATPase activity in growing plants showed a small decline after 2 days of 0.5 ppb treatment and increased in plants treated for 8 days with HF. Dormant plants also showed an increase in ATPase activity when fumigated with HF for 28 days. These results suggest that the plasma membrane lipid composition in seedlings was changed very early in response to F treatment. It is possible that these changes in the lipid environment of the plasma membrane ATPase could protect the activity of this enzyme against F. Sterols have been demonstrated to activate plasma membrane ATPase in plants (Grandmougin et al. 1989a,b, Sandstrom and Cleland 1989). It is also plausible that the changes in the plasma membrane lipids, which may be required to protect the activity of ATPase, have a negative effect on plasma membrane permeability. Increased sterol:phospholipid ratios rigidify cell membranes (Anderson et al. 1981) and promote membrane leakiness (Shinitzky 1984). In our earlier study (Zwiazek and Rakowski 1992), we observed increased leakage of electrolytes from eastern white pine shoots after 2 days of

fumigation with 0.5 ppb and 2 ppb HF. The same F treatments drastically increased sterol:phospholipid ratios in the present study.

Another explanation for the observed early increase in sterol:phospholipid ratios followed by the increase in ATPase activity in the plasma membranes may be that only visibly undamaged plants were used for the experiments. The first signs of visible injury in seedlings treated with 0.5 ppb HF and 2 ppb HF were observed after 20 days and 5 days respectively. Therefore, the increase in sterol:phospholipid ratios of the plasma membranes may be among the first symptoms leading to visible needle injury and the increased activity of ATPase may represent a defense mechanism present in F-resistant seedlings only.

The results of our study indicate that dormant plants respond in a similar manner to actively growing plants, but more time is needed for F to affect the plasma membranes. This is in agreement with an earlier study (MacLean et al. 1986) that showed that dormant trees are less sensitive to pollution than actively growing trees.

In the earlier study conducted with jack pine treated with sodium fluoride (Zwiazek and Shay 1987) we noted profound changes in cell structure. However, many of these alterations, including the accumulation of lipid droplets near cell membranes, could not be separated from the effects of water stress induced by sodium fluoride. In the present study we did not observe any significant alterations in cell structure at the time when the changes in the composition of the plasma membranes were apparent. Therefore, the accumulation of lipid droplets in cells may represent an effect of water stress rather than a direct fluoride effect.

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Figure 1. Sterol:phospholipid ratios in actively growing (a) and dormant (b) eastern white pine seedlings subjected to HF treatments. Bars indicate means (n=3) + SE.

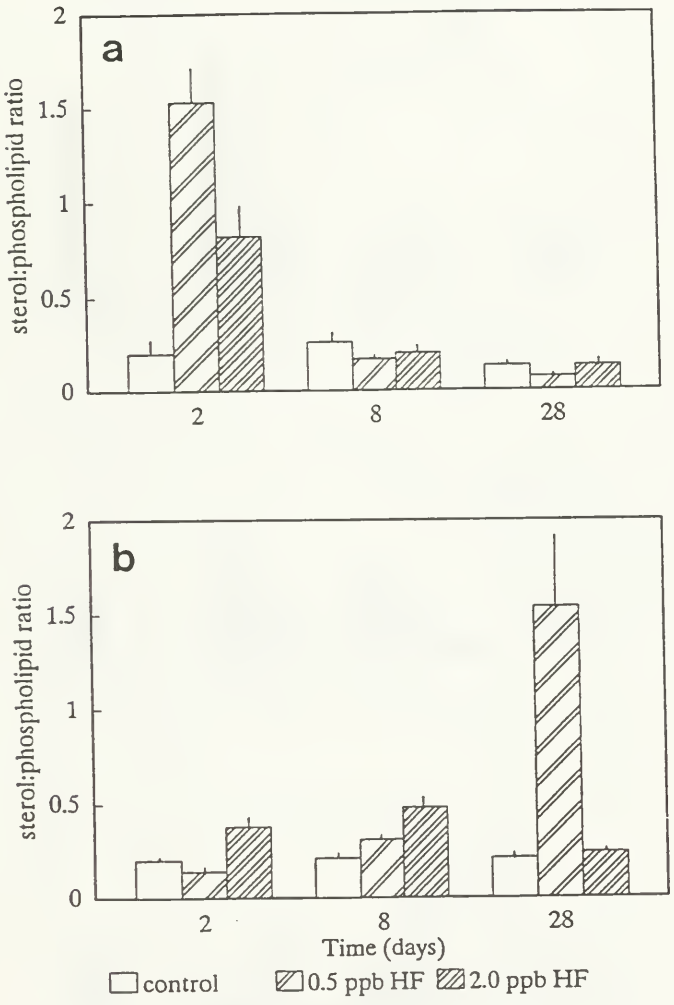

Figure 2. Effect of HF on sterol composition in the plasma membranes of actively growing eastern white pine seedlings treated with HF for 2 days (a), 8 days (b) and 28 days (c). Means (n=3) + SE are shown.

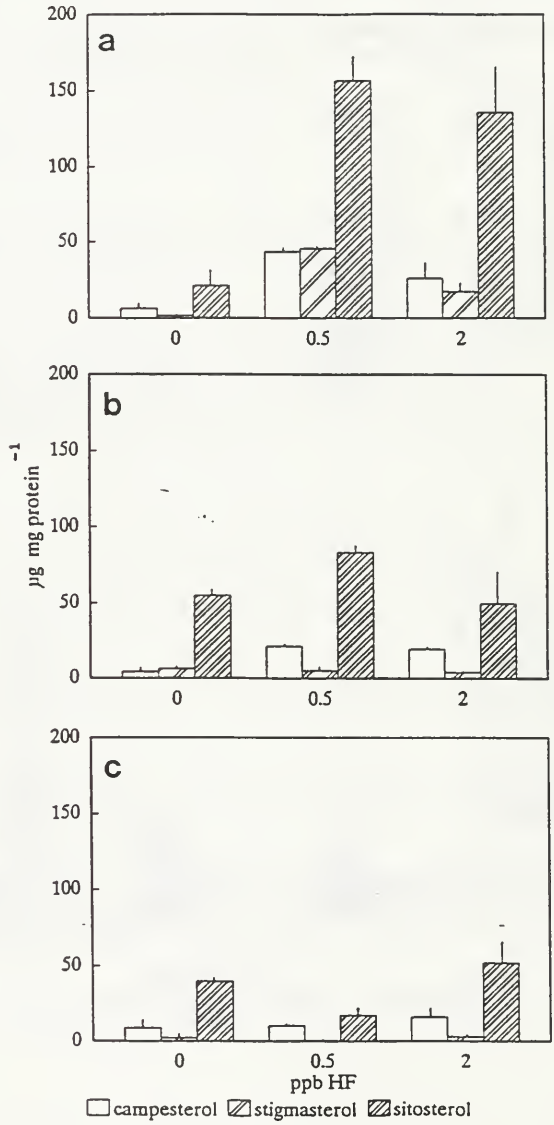

Figure 3. Effect of HF on sterol composition in the plasma membranes of dormant eastern white pine seedlings treated with HF for 2 days (a), 8 days (b) and 28 days (c). Means (n=3) + SE are shown.

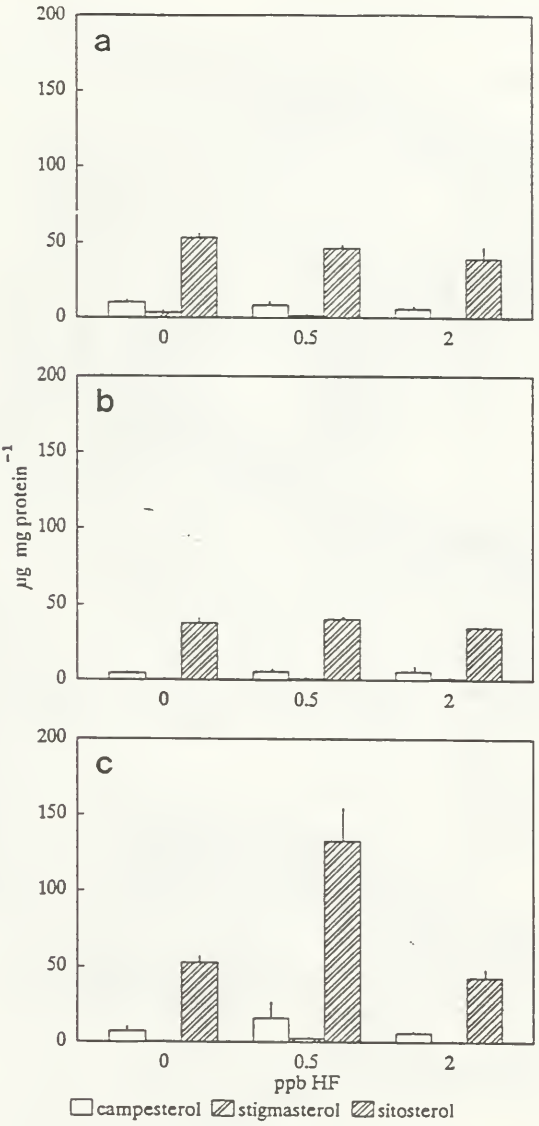

Figure 4. Effect of HF on plasma membrane ATPase activity in actively growing (a) and dormant (b) eastern white pine seedlings. Means (n=4) + SE are indicated.

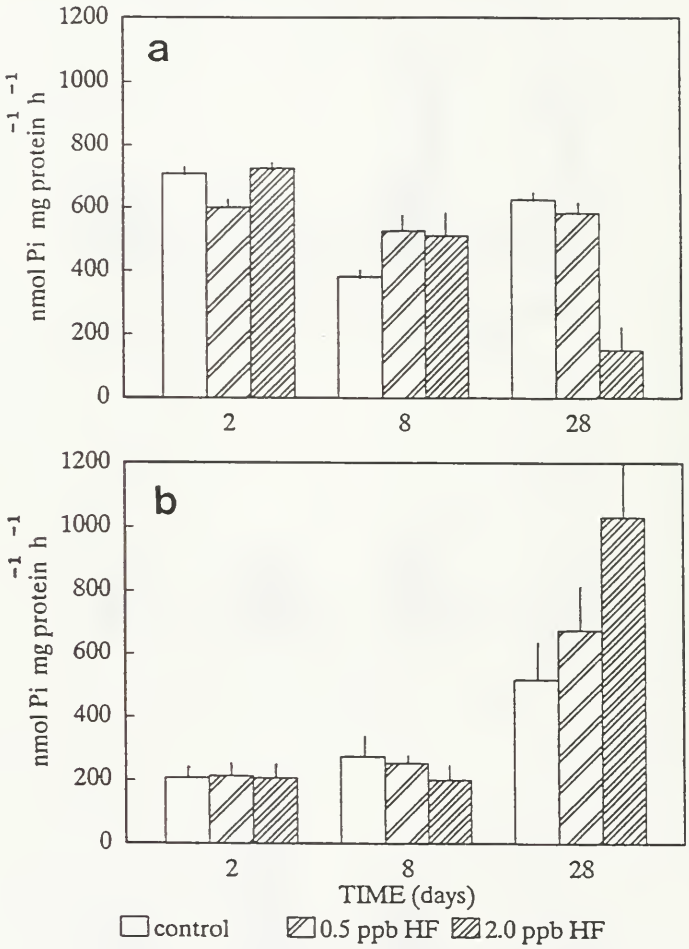

Figure 5. Needle structure of actively growing control (a) eastern white pine seedlings and seedlings subjected to 0.5 ppb HF for 23 days (b) and 2 ppb HF for 7 days (c).

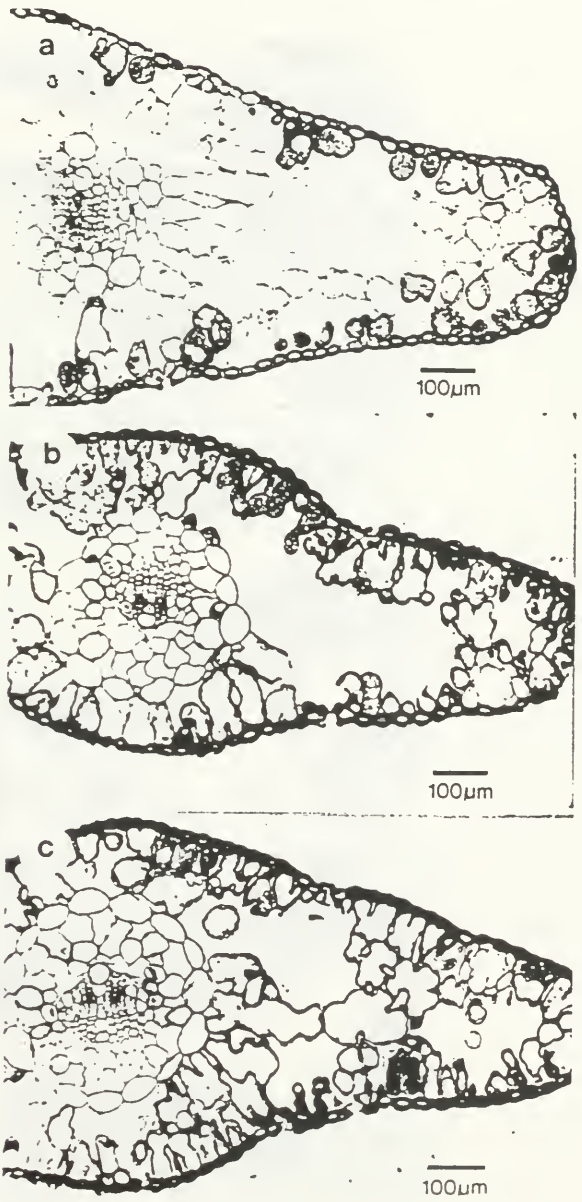
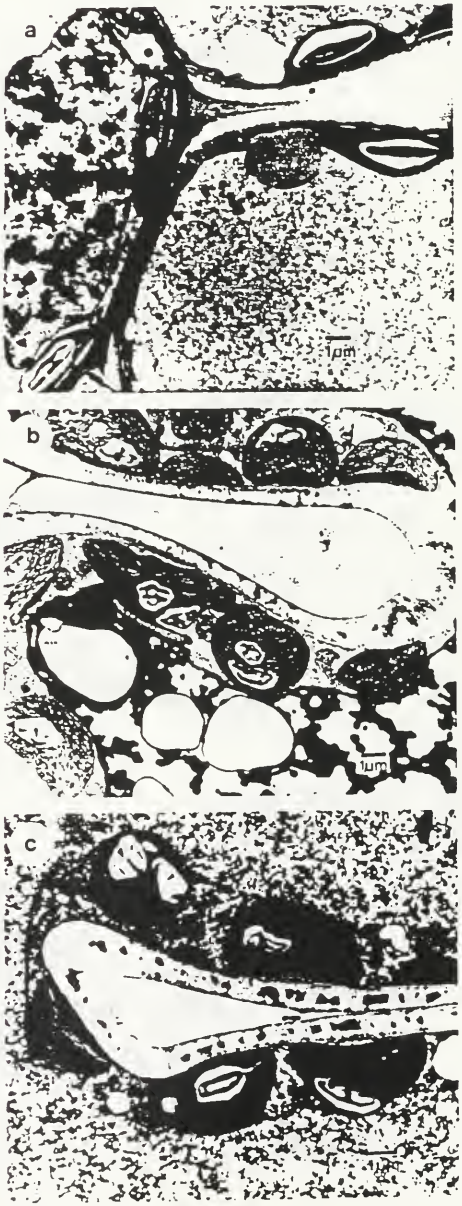
Figure 6. Ultrastructure of mesophyll cells in actively growing control (a) eastern white pine seedlings and seedlings treated with 0.5 ppb HF for 23 days (b) and 2 ppb HF for 7 days (c).